Oxidation Products of Amino Acids and Collagen

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Amino acids are known to react with oxidizing agents and some of the degradation products are less complex amino acids. In this study, 14 amino acids as well as fibrous corium collagen and epoxy resin-tanned collagen were reacted with a hydrogen peroxide or hydrogen peroxide–copper sulfate solution, and a number of amino acids were identified chromatographically which were not originally present. These results may explain the presence of several ninhydrin-reactive unknowns formed during hydrolysis of the product obtained by tanning fibrous corium collagen with an epoxy resin.

Epoxy resin has been shown to impart a very stable tannage to fibrous corium collagen (1). This tanned collagen is resistant to enzymatic degradation and cannot be partially decomposed to obtain smaller fragments for further study. In attempting to identify the stable linkages formed, the cupric ion–hydrogen peroxide method of solubilizing and degrading collagen and leathers used by Deasy (2–6) was employed.

The degradation of specific amino acids has been studied by others. Matsuo (7) degraded methionine using hydrogen peroxide and found several products including methionine sulfone which we have identified in peroxide-degraded collagen.

The photooxidation of tryptophan was studied by Asquith and Rivett (8). They concluded that the primary reaction yields formylkynurenine, while secondary reactions result in the formation of aspartic acid, serine, glycine, and β-alanine.

Arlinghaus (9) reported the isolation of 3-hydroxyproline and found it could be oxidized to β-alanine by treatment with potassium permanganate.

In our work we have followed the degradation of the higher molecular weight amino acids through several steps to lower molecular weight amino acids. Examples of this phenomenon are the production of β-alanine from ornithine and also from 4-aminobutyric acid, which is an oxidation product of ornithine.

A carbon atom rearrangement may be involved in the oxidative dea-

[1] Agricultural Research Service, U.S. Department of Agriculture.

mination of arginine as the chain is slowly broken down to form a number of intermediates. It should be pointed out that the identification of the amino acids is based entirely on chromatographic evidence, therefore it is possible that some of the peaks identified as amino acids may be unknowns since they have not been characterized by other methods. Other oxidative systems both *in vivo* and *in vitro* might also alter the amino acid analyses of proteins.

MATERIALS

All of the amino acids were chromatographically pure and were obtained from commercial sources.

The hydrogen peroxide used was prepared in distilled water by dilution of J. T. Baker Chemical Company's[2] unstabilized 30% hydrogen peroxide. The hydrogen peroxide and cupric ion–hydrogen peroxide solutions were prepared immediately before use. The cupric sulfate was analytical reagent grade.

PROCEDURES

The peroxide degradation procedure was similar to that used by Deasy (6). An 18 ml aliquot of a 5.5 or 11 mM solution of an amino acid and 2 ml of 30% hydrogen peroxide were mixed in a 30 ml beaker. The beaker was covered with a watch glass and the solution heated in an oven at 80–85°C for various periods of time. The sample was then cooled and platinum grids plated with platinum black were added to break down the excess peroxide. The samples were made up to 25 ml with water prior to amino acid analysis.

A few amino acids were subjected to a more drastic oxidation procedure using a 3% hydrogen peroxide and 5×10^{-4} M cupric sulfate solution (3). After the oxidation, the copper was precipitated with hydrogen sulfide and filtered off prior to running the samples on the amino acid analyzer.

One milliliter of the oxidized amino acid solution was diluted to 5 ml using 0.1 N hydrochloric acid and 1 ml of this dilution was run on a Piez-Morris (10) ion-exchange column employing a continuous gradient elution buffer. The 140 cm column at 60°C is capable of separating all of the common amino acids on a 24-hr run.

The method of preparation of epoxy-tanned fibrous corium collagen used for our experiments has been described (1). Samples of fibrous corium collagen and epoxy-tanned collagen were degraded according to the method of Deasy (3) using a 3% hydrogen peroxide and 5×10^{-4} M cupric sulfate solution.

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

The samples of collagen were hydrolyzed by refluxing in 6 N hydrochloric acid for 18 hr. The products were evaporated to dryness on a steam bath under a current of nitrogen gas and then further evaporated three successive times from distilled water solutions to expel hydrochloric acid. The residues were dissolved in 0.1 N hydrochloric acid to prepare the solutions for amino acid analysis.

An attempt was made to prepare a derivative of proline and epoxy resin on a mole for mole basis using the same conditions employed for the epoxy resin-tannage of fibrous corium collagen (1). The reacted solution was evaporated to dryness and the proline–resin compound was extracted from the sodium carbonate buffer salt with methanol. The material was run on the amino acid analyzer both before and after acid hydrolysis.

In an effort to identify as many unknown peaks as possible, a number of amino acids not usually found in collagen, but possible products of oxidation, were run on the amino acid analyzer. These included: β-alanine, citrulline, pipecolinic acid, α-aminoadipic acid, 4-aminobutyric acid, α-aminoisobutyric acid, α-amino-*n*-butyric acid, norvaline, 5-amino-*n*-valeric acid, and ornithine. Most of these had originally been identified by Zacharius and Talley (11) using a two-column system. Possible intermediates which were also degraded with hydrogen peroxide are: 4-aminobutyric acid, β-alanine, α-aminoadipic acid, and ornithine.

RESULTS AND DISCUSSION

Peroxide Oxidation of Amino Acids

Amino acids that were reacted with hydrogen peroxide are shown in Table 1. The locations of the unknown peaks and other degradation products are shown in Fig. 1. All of the unknown peaks are detected at 570 nm except C which is detected at 440 nm. In all but two of the amino acids there is a reduction in chain length for the oxidized product. The exceptions to this are the formation of hydroxylysine from lysine and 3-hydroxyproline from proline. The amount of hydroxylysine produced in the 4-hr reaction period of lysine oxidation is greater than for the 1-hr period. Ornithine is apparently oxidized on the second carbon converting this to a carboxyl, forming 4-aminobutyric acid. Further oxidation of the 4-aminobutyric acid produces β-alanine, apparently indicating a chain-shortening process by oxidation from the carboxyl end of the molecule.

When arginine is reacted with 3% hydrogen peroxide solution for 4 hr at 80–85°C, 52% of the arginine remains and a peak where lysine elutes is present which accounts for 22% of the starting material on a molar basis when calculated as lysine. There are also traces of material where

TABLE 1

OXIDATION OF AMINO ACIDS

Amino acid	Reaction time (hr)	% Recovery	Other products and yield[a]
Arginine	4	52	NH_3, Lys(22), Asp(tr), Glu(tr), J(tr), K(tr)
Lysine	1	85	NH_3, Hyl(tr)
	4	35	NH_3, Hyl(10), β-ala(1), I(12)
Hydroxylysine	4	47	NH_3, Asp(tr), Glu(tr), Gly(tr), β-Ala(tr), A(tr), H(tr)
Ornithine	2	42	NH_3, β-Ala(2), 4-NH_2-but(7), D(tr)
4-Aminobutyric acid	4	14	NH_3, β-Ala(6), D(3)
Glutamic acid	2	91	NH_3, Asp(tr)
Aspartic acid	4	88	NH_3, Gly(tr), F(tr)
β-Alanine	4	76	NH_3
Glycine	1	99.8	
	4	24	NH_3
Tyrosine	4	59	NH_3, B(tr)
Leucine	4	8	NH_3, L(7)
Valine	4	46	NH_3, Thr(tr)
α-Aminoadipic acid	4	tr	NH_3, C(tr), G(8)

[a] Yields of the amino acids are given in parentheses as mole percent of the oxidized amino acid; tr indicates a trace too small to calculate as a definite yield figure. The letters identify unknown compounds shown on the chromatogram in Fig. 1.

aspartic acid and glutamic acid elute in addition to two unknowns and ammonia.

The possible degradation reactions may be a rearrangement of the guanidino group to eliminate two nitrogens and couple the guanidino carbon to the end of the main carbon chain producing lysine. About 46% of the oxidized arginine appears to follow this reaction path. A small amount of the arginine is apparently oxidized on carbon 5 to

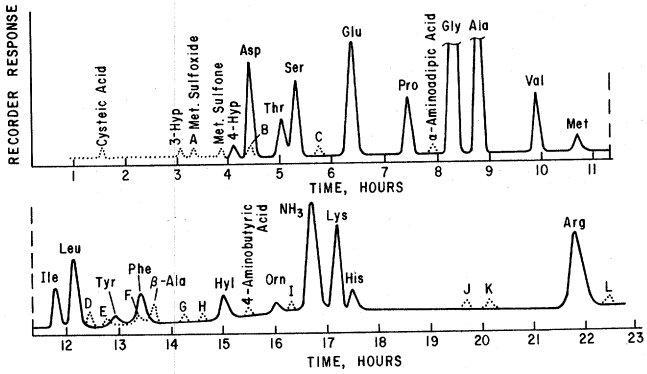


FIG. 1. Chromatogram showing elution times of amino acids from a collagen hydrolysate (solid-line curve) and oxidation products of amino acids and collagens (broken-line curve).

produce glutamic acid which can be further oxidized to produce aspartic acid. Aspartic acid has been oxidized to yield ammonia and a small amount of glycine, and the glycine can be oxidized to ammonia. This second degradation pathway for arginine is completely different from the first pathway to lysine because lysine has been found to oxidize to hydroxylysine and β-alanine but did not form any glutamic or aspartic acids or glycine, which can be formed from aspartic acid.

The oxidation of ornithine for 2 hr caused a loss of 58% of the ornithine and the formation of 4% of β-alanine and 7% of 4-aminobutyric acid on a molecular basis. The 4-aminobutyric acid has been oxidized to β-alanine which can be further oxidized to ammonia.

When valine is oxidized, apparently the first step is the oxidation of one of the terminal methyl groups to produce a hydroxyl on the backbone chain. This threonine oxidizes easily to yield ammonia as the final nitrogenous product.

α-Aminoadipic acid was considered as a possible oxidation product of lysine and, when oxidized with hydrogen peroxide solution for 4 hr, yields two unknowns. One of these has been found in the hydrolyzed residue from peroxide-treated, epoxy-tanned collagen as well as in an epoxy lysine preparation which was also hydrolyzed and run on the amino acid analyzer.

Peroxide Oxidation of Proline

The rate of oxidation of proline was studied using 10-min, 1-, 4-, and 8-hr reaction times. For the 10-min experiment, all of the proline was recovered. The proline recoveries for the other intervals as well as recoveries for other ninhydrin-reactive products formed can be found in Table 2.

Peroxide oxidation of proline appears to involve hydroxylation, forming 3-hydroxyproline as the first step. This has been identified by amino acid analysis. A peak produced by reaction with ninhydrin and measured at 440 nm was present on the chromatogram at the same elu-

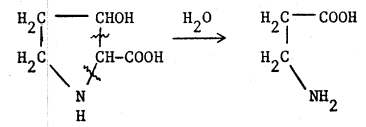
TABLE 2
OXIDATION OF PROLINE

Reaction time (hr)	Recoveries (percent on molar basis)					
	Proline	3-Hyp[a]	β-Ala[b]	4-Aminobutyric acid	NH_3	Total
1	71.4	14.5	trace	1.5	6.8	94.2
4	60.6	15.0	2.8	2.4	25.0	105.8
8	32.5	9.0	4.7	1.9	29.1	77.2

[a] 3-Hydroxyproline.
[b] β-Alanine.

tion time as the peak identified by Piez *et al.* (12) as 3-hydroxyproline. The method of amino acid analysis was the same. The amount present was calculated using the constant for 4-hydroxyproline since 3-hydroxyproline was not available.

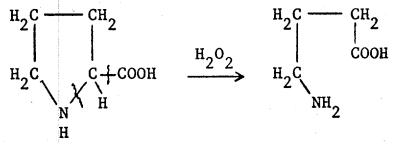
Further evidence that the compound is 3-hydroxyproline is based on the continuing oxidation of proline and 3-hydroxyproline. 3-Hydroxyproline is produced in the first stages of the reaction; then as it decreases, there is a rise in the amount of β-alanine. Arlinghaus (9) and Ogle *et al.* (13) have isolated 3-hydroxyproline and oxidized it with $KMnO_4$ obtaining β-alanine. They concluded that the β-alanine arises from the following reaction:



3-hydroxyproline β-alanine

When β-alanine was added to a portion of the oxidized proline sample, the unknown peak and β-alanine gave a symmetrical peak on the amino acid analyzer.

4-Aminobutyric acid is indicated as another degradation product of proline oxidation since a peak at its characteristic location is present and reaches a maximum height at 4 hr of oxidation.



proline 4-aminobutyric acid

When 4-aminobutyric acid is oxidized with hydrogen peroxide solution, the products are ammonia, β-alanine, and one unidentified substance which is also present in the greatest amount in the 4-hr-oxidized proline sample. This unknown, D, has also been found in peroxide-degraded collagen. These results indicate the β-alanine produced by oxidizing proline may come from two intermediates, 3-hydroxyproline and 4-aminobutyric acid.

In addition to these products of proline oxidation, there were also traces of two unknowns, D and E, plus 0.1% glycine for the 4-hr experiment and 0.2% glycine for the 8-hr experiment. A trace of material where aspartic acid elutes was also seen on the chromatograms for the 4- and 8-hr experiments.

The amino acid analysis of the proline–resin derivative gave the same results for both hydrolyzed and unhydrolyzed samples. There was no proline and no other peaks except for ammonia. This would indicate that

any derivative which may have formed is extremely stable to 6 N hydrochloric acid-hydrolysis and does not react with ninhydrin, or the proline has been completely degraded by the action of the epoxy resin.

Peroxide–Copper Sulfate Oxidation of Amino Acids

As seen in Table 1, the rate of oxidation varies for the different amino acids using a 3% hydrogen peroxide solution at 80–85°C. Using the peroxide–copper sulfate oxidation procedure, proline produced 12.8% glycine and 5.3% β-alanine on a molar basis with no proline recovered after reacting for 1 day at room temperature. After 5 days, 0.4% glycine and 2.4% β-alanine were recovered. In both cases there was a large peak for ammonia.

Other amino acids that were reacted with the hydrogen peroxide–cupric ion solutions for 5 days included tyrosine, phenylalanine, histidine, valine, isoleucine, and leucine. In all cases, none of the original amino acids were recovered, but a large ammonia peak and a small glycine peak were present. When glutamic acid was reacted under the same conditions for 1 day, the glutamic acid was destroyed and in addition to the large ammonia peak and small peak for glycine, there were traces of material where aspartic acid and serine elute.

To determine the effect of cupric ion on the rate of oxidation of amino acids, leucine was oxidized using a 3% hydrogen peroxide and 5×10^{-4} M copper sulfate solution at 80–85°C for 15-, 30-, and 45-min intervals. The amount of leucine recovered was 48% from the 15-min sample, 5% from the 30-min sample, and 2% from the 45-min sample. These results can be compared with the leucine oxidation using 3% hydrogen peroxide at 80–85°C for 4 hr without any copper ions. Under these conditions, 8% of the leucine was recovered.

Peroxide–Copper Sulfate Oxidation of Collagens

In the degradation study of collagens, 1 gm of hide collagen in 25 ml of solution had solubilized in 4 days, whereas after 1 week only about 50% of the epoxy-tanned collagen had solubilized. The only free amino acid present in the solubilized collagen was a small amount of glycine. The copper in the soluble fraction was precipitated with hydrogen sulfide and the soluble and insoluble fractions were hydrolyzed with 6 N hydrochloric acid and the amino acids determined on the hydrolysates. The results are shown in Table 3. Hydrolyzed hide collagen is also shown for comparison. Methionine, tyrosine, histidine, and cystine have disappeared from all of the fractions. Phenylalanine is present in an appreciable amount only in the insoluble fractions and these have a higher amount of proline and no β-alanine. It is not surprising that the hydroxylysine and lysine values are substantially lower in all of the

TABLE 3
OXIDATION OF COLLAGENS

Amino acid	Percent μmoles					
		H_2O_2 epoxy collagen				
		Soluble		Insoluble		
	H_2O_2 collagen	1 wk	2 wk	1 wk	2 wk	Collagen[a]
Hydroxyproline	8.1	7.2	6.5	8.9	8.4	8.8
Aspartic acid	6.0	6.6	7.8	6.0	6.7	5.0
Threonine	1.4	1.5	1.6	1.7	1.8	1.7
Serine	3.1	3.2	3.2	3.6	3.7	3.3
Glutamic acid	9.3	9.2	10.3	8.9	9.8	7.3
Proline	14.1	10.6	14.8	16.2	15.7	12.8
Glycine	35.5	40.1	32.8	30.3	30.8	32.8
Alanine	10.8	12.7	13.9	12.8	11.1	10.5
Valine	1.8	2.1	2.1	2.1	2.2	2.3
Isoleucine	0.9	1.0	1.0	1.2	1.2	1.3
Leucine	1.8	1.7	1.6	2.2	2.3	2.6
Tyrosine	none	none	none	none	none	0.5
Phenylalanine	none	trace	trace	1.2	1.2	1.4
Hydroxylysine	0.5	none	trace	trace	trace	0.7
Lysine	2.1	0.2	0.2	0.2	0.2	2.6
Histidine	none	none	none	none	none	0.4
Arginine	4.8	4.1	4.1	4.8	4.9	5.4
Methionine sulfone	*[b]	*	*	*	*	
4-Aminobutyric acid	*	*	*	*	*	
Methionine sulfoxide	*		*			
β-Alanine	*	*	*			
Cysteic acid	*					
Unknowns (number)	1	2	6	2	2	2

[a] Native collagen also contains ornithine, cystine, and methionine.
[b] Indicates presence.

epoxy-tanned collagen fractions since these contained less of these amino acids before reacting with the hydrogen peroxide. The increased amounts of aspartic acid, glutamic acid and glycine found in the peroxide-treated collagens may be attributed to the functioning of the oxidative degradation system since these amino acids have been shown to be produced from larger amino acids.

CONCLUSIONS

1. It has been demonstrated that when amino acids are reacted with a hydrogen peroxide solution, the ninhydrin-reactive compounds formed by the oxidative degradation are usually less complex amino acids and ammonia.

2. Several examples of shortening of chain length were observed in the oxidative degradation of amino acids.

3. The study of the oxidation products of amino acids has helped to elucidate the mechanism of epoxy resin tannage of fibrous corium collagen by identifying some of the previously unknown ninhydrin-reactive products formed during hydrolysis of the tanned product.

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